Final Technical Report
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Principal Investigator: Ruth Globus, Ph.D.

Mechanical forces generated by gravity, weightbearing, and muscle contraction play a key role in the genesis and maintenance of skeletal structure. Increased mechanical loading caused by exercise stimulates osteoblasts resulting in increased bone formation and accretion of skeletal mass. Conversely, astronauts exposed to prolonged space flight suffer from site-selective osteopenia, which has been shown in growing rats to result from reduced bone formation by osteoblasts. The reduction in bone formation appears to be caused by defects at several stages of osteoblast differentiation, including proliferation, matrix production, and mineralization. The molecular mechanisms that mediate changes in osteoblast activity in response to altered patterns of skeletal loading are not known, and a better understanding of these processes may be essential for developing effective treatment strategies to prevent disuse osteoporosis.

The long-term goal of our collaborative research program is to understand how the extracellular matrix (ECM) and cell adhesion proteins, integrins, interact to mediate the response of osteoblasts and their progenitors to mechanical loading. We suggest that integrin/ECM interactions are crucial for basic cellular processes, including differentiation and survival, as well as to participate in detecting and mediating cellular responses to mechanical stimuli.

Major Findings

As a first approach to determine the role of integrin/extracellular matrix (ECM) interactions in bone formation, we analyzed the repertoire of integrins expressed in bone and the influence of integrin/ECM perturbing factors on osteoblast differentiation *in vitro*. Osteoblasts in fetal rat calvaria, and cultures derived from this tissue, express a large number of different integrin receptors for fibronectin, collagen, and other ECM ligands, including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha8\beta1$, $\alpha V\beta3$ and $\alpha V\beta5$.

Integrin complexes detected on rat calvarial cells in vivo and in vitro and their ECM ligands

$\alpha1\beta1$	collagen, laminin
$\alpha2\beta1$	collagen, laminin
$\alpha3\beta1$	laminin, fibronectin, thrombospondin, collagen
$\alpha5\beta1$	fibronectin
$\alpha8\beta1$	fibronectin, vitronectin, tenascin
$\alpha V\beta3$	vitronectin, fibronectin, fibrinogen, osteopontin, bone sialoprotein
$\alpha V\beta5$	vitronectin, fibronectin

Primary cultures of fetal rat osteoblasts progressively differentiate in culture to form mineralized nodules . To dissect the role of specific ECM proteins, we added function-perturbing antibodies and fragments of ECM ligands to primary osteoblasts at different stages of differentiation. We found that the addition to immature osteoblasts of antibodies to $\alpha3\beta1$, $\alpha5\beta1$, and $\alpha8\beta1$ (but not $\alpha V\beta3$, $\alpha V\beta5$, $\alpha4\beta1$), as well as soluble ligands (fibronectin, laminin), ligand antibodies, and RGD peptides (which interfere with integrin/ECM binding), inhibits both the expression of genes characteristic of the osteoblast phenotype and the formation of mineralized nodules .

To determine if FN also plays an important role in the function of mature osteoblasts as well as during differentiation, osteoblasts that had already formed mineralized nodules *in vitro* were treated with FN antagonists FN antibodies (FNAb) caused >95% of the cells in mature cultures to display characteristic features of apoptosis within 24 h (nuclear condensation, apoptotic body formation, DNA laddering). Cells appeared to acquire sensitivity to FNAb-induced apoptosis as a consequence of differentiation, since FNAb failed to kill immature cells and the first cells killed by FNAb were those associated with mature nodules. Intact plasma FN, as well as fragments corresponding to the amino-terminal, cell-binding, and carboxy-terminal domains of FN, independently induced apoptosis of mature, but not immature, osteoblasts. Thus FN appears to function in the mature, but not the immature, ECM to sustain osteoblast survival. Finally, transforming growth factor-ß1 partially protected cells from the apoptotic effects of FNAb, indicating that TGF-ß may function in concert with FN, to promote osteoblast survival *in vivo*. We conclude that FN functions to promote survival of osteoblasts once they have matured, and that this may contribute to the regulation of bone formation. Interestingly, the same anti-integrin antibodies and RGD peptides that inhibit differentiation of immature cells fail to induce apoptosis of mature osteoblasts. Thus, mature osteoblasts appear to rely on multiple interactions with the ECM to ensure their survival *in vitro*.

Summary: Influence of Integrin/ECM Antagonists on Osteoblast Differentiation and Survival (see references below)

Additive:	Differentiation of immature Ob	Survival of mature Ob
FN Antagonist (Ab, FN)	↓	↓
LN Antagonist (Ab, LN)	↓	↓
RGD Peptide	↓	↔
α3β1 Ab	↓	↔
α5β1 Ab	↓	↔
α8β1 Ab	↓	↔
αVβ3 Ab	↔	?
αVβ5 Ab	↔	?

To evaluate the effects of mechanical strain applied *in vitro,* studies were performed using a commercially-available apparatus (Flexercell strain unit) using Flexcell flexible dishes for both control and mechanically active conditions to generate both compressive and tensile strains on primary osteoblasts. The expression of specific ECM, integrin and cytoskeletal components in response to mechanical forces (4% maximum deformation, 0.5 cycles/sec) was analyzed using immunocytochemical techniques. We found that consistent changes in the pattern of expression of fibronectin, α5 integrin, and actin are not evident after 2hr, 2d or 4d of strain relative to stationary controls. Since the mechanical strain provided by the Flexcell system is non-uniform and provides strain levels that greatly exceeded physiologic levels for bone we developed a novel strain unit that can: 1) apply a predominantly uniaxial and uniform load to the culture substrate at strains thought to be physiological for bone *in vivo* 2) dynamically load cells with cyclic tensile and compressive mechanical deformation 3) permit real-time microscopic observations by confocal imaging, without interrupting the loading

cycle. This loading unit is useful for evaluating integrin-ECM interactions that mediate cellular responses to mechanical strain.

In conclusion, we have made substantial progress in elucidating those integrin/ECM interactions that are needed for osteoblast function and have developed a useful loading system to further explore the molecular basis of mechano-sensitivity of osteoblasts.

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Ruth Globus, Ph.D.

Caroline Damsky, Ph.D.

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Nasa Ames Research Center
mail Stop 236-7
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